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Rubella virus capsid protein modulation of viral genomic and subgenomic RNA synthesis

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Abstract

The ratio of the subgenomic (SG) to genome RNA synthesized by rubella virus (RUB) replicons expressing the green fluorescent protein reporter gene (RUBrep/GFP) is substantially higher than the ratio of these species synthesized by RUB (4.3 for RUBrep/GFP vs. 1.3–1.4 for RUB). It was hypothesized that this modulation of the viral RNA synthesis was by one of the virus structural protein genes and it was found that introduction of the capsid (C) protein gene into the replicons as an in-frame fusion with GFP resulted in an increase of genomic RNA production (reducing the SG/genome RNA ratio), confirming the hypothesis and showing that the C gene was the moiety responsible for the modulation effect. The N-terminal one-third of the C gene was required for the effect of be exhibited. A similar phenomenon was not observed with the replicons of Sindbis virus, a related Alphavirus. Interestingly, modulation was not observed when RUBrep/GFP was co-transfected with either other RUBrep or plasmid constructs expressing the C gene, demonstrating that modulation could occur only when the C gene was provided in *cis*. Mutations that prevented translation of the C protein failed to modulate RNA synthesis, indicating that the C protein was the moiety responsible for modulation; consistent with this conclusion, modulation of RNA synthesis was maintained when synonymous codon mutations were introduced at the 5′ end of the C gene that changed the C gene sequence without altering the amino acid sequence of the C protein. These results indicate that C protein translated in proximity of viral replication complexes, possibly from newly synthesized SG RNA, participate in regulating the replication of viral RNA.

Keywords: Rubella virus; Genomic RNA synthesis; Subgenomic RNA synthesis

Introduction

The genome of rubella virus (RUB) is a single-stranded RNA of plus polarity, ~10,000 nt in length, that contains two open reading frames (ORF's). The 5′ proximal ORF encodes two nonstructural proteins involved in virus RNA replication while the 3′ proximal ORF encodes the three virion proteins, the capsid protein C and envelope glycoproteins E1 and E2 (the gene order is 5′-C-E2-E1-3′ within the ORF). These ORF's are designated the nonstructural protein ORF (NS-ORF) and structural protein ORF (SP-ORF), respectively, and the NS-ORF is translated from the genome RNA while the SP-ORF is translated from a subgenomic (SG) RNA consisting of roughly the 3′ third of the genomic RNA. Both RNA species are transcribed from a genome-length RNA of minus polarity in infected cells. More SG than genome RNA is synthesized; the molar ratio of SG to genome RNA synthesized as determined by [^{3}H]-uridine incorporation was ~1.6 (Hemphill et al., 1988).

Defective-interfering (DI) RNAs of RUB generated during serial passage contain large internal deletions in the SP-ORF but generally maintain the 5′ end of the C gene and always maintain the 3′ end of the E1 gene (Derdeyn and Frey, 1995; Frey and Hemphill, 1988). These DI RNAs can replicate and direct synthesis of a truncated SG RNA (Derdeyn and Frey, 1995; Tzeng et al., 2001). Recently, it has been found that the SP-ORF can be replaced with a reporter gene to generate a "replicon" that replicates intracellularly and expresses the reporter gene but does

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not spread from cell to cell unless wt RUB is present to provide the virion proteins (Tzeng et al., 2001). RUB replicons thus resemble RUB DI RNAs and their genetic structure demonstrates that the 5′ end of the C gene is not necessary for replication of either species. However, we have noted that replicons synthesize little genome RNA relative to SG RNA (Tzeng and Frey, 2002).

Recent characterization of the RUB C protein has revealed that it is phosphorylated at a single site and that phosphorylation/dephosphorylation of this site is important in genome RNA binding and in encapsidation during virion formation (Law et al., 2003). The C protein was also found to bind to a mitochondrial protein, although the function of this binding in the virus replication cycle has not been elucidated (Beatch and Hobman, 2000; Mohan et al., 2002). We recently found that the C protein can complement a 500 nt deletion in the NS-ORF (Tzeng and Frey, 2003) and it was also reported that the C protein increases the replication efficiency of RUB mutants in the 3′ *cis*-acting sequences and poorly replicating vaccine strains (Chen and Icenogle, 2004). In the course of these experiments, a series of C protein-reporter protein fusions was generated that contained increasing N-terminal regions of the C protein. We noticed that replicons expressing C-reporter protein fusions with ~>30% of the C protein synthesized markedly increased levels of genome RNA relative to the SG RNA. In this study, we have expanded that observation and show that expression of the C gene is necessary for synthesis of the genome/SG RNA ratio observed in RUB-infected cells.

Results

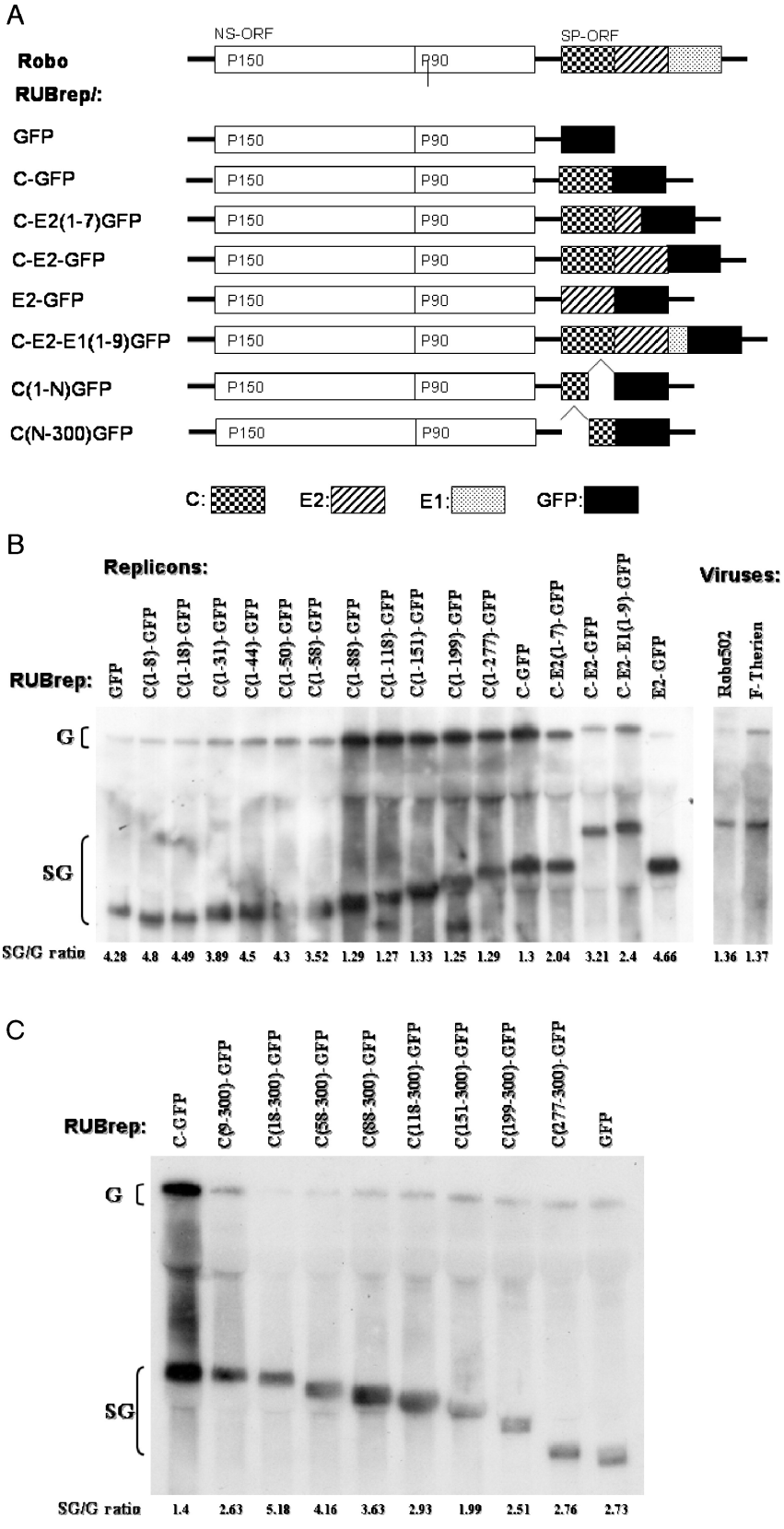
Expression of the C gene enhances replicon genomic RNA synthesis

In previous studies, the relative amounts of virus-specific genomic and SG RNA on Northern blots of RNA from RUB-infected cells appeared approximately equal and the molar ratio of SG to genomic RNA synthesis determined by metabolic radiolabeling over a 12-h period was found to be 1.6 (Hemphill et al., 1988). However, in Northern blots of RNA from RUBrep/GFP-transfected cells (genomic diagrams of the infectious cDNA clone and replicon constructs are in Fig. 1A), the genomic RNA band was faint relative to the SG RNA band (Tzeng and Frey, 2002), as shown in Fig. 1B; as the GFP gene was used as a probe, the band intensities correlate directly with molar amounts of the genomic and SG RNAs produced. The only difference between virus genome and RUBrep/GFP is the SP-ORF. Therefore, we hypothesized that a structural gene or protein could modulate RNA synthesis. To this end, a series of replicons were constructed that contained in-frame fusions of individual structural proteins with GFP (Fig. 1A). As shown in Fig. 1B, in cells transfected with RUBrep/C-GFP, a replicon expressing the C protein fused in-frame with GFP, the genomic RNA band was markedly more intense. The ratio of the intensities of the SG/genomic RNA bands was 4.3 for RUBrep/GFP, but 1.3 for RUBrep/C-GFP, comparable to the SG/genomic RNA ratio of 1.3–1.4 in RUB-infected cells (which is in good agreement with the synthesis rate determined by metabolic labeling). In contrast, RUBrep/E2-GFP, which expresses an E2-GFP fusion protein, produced SG and genomic RNA at a ratio of 4.7. Thus, the C gene is the moiety responsible for increase of genomic RNA production relative to SG RNA. In RUBrep constructs that expressed fusion proteins containing C and other regions of the SP-ORF [C-E2(1-7)-GFP, C-E2-GFP, or C-E2-E1(1-9)-GFP, the numbers indicate the amino acid residues included in the fusion], the SG/genomic RNA ratio was 2–3, intermediate between the ratio produced by RUBrep/GFP and RUBrep/C-GFP, suggesting that in the replicon context, the C gene is most effective at increasing the relative level of genomic RNA when expressed alone [the rubella virion proteins mature as a complex (Law et al., 1999) and presence of the GFP gene fused to the envelope glycoproteins could interfere with the normal maturation of this complex].

Next, to examine whether the complete C gene or only part would suffice to modulate viral RNA synthesis, a series of C-GFP fusions that contained progressive deletions of the C gene from either the 5′ or 3′ end were generated. A series of 11 RUBrep constructs that expressed a nested set of progressively increasing N-terminal regions of the C protein fused with GFP [C(1-8)- through C(1-277)-GFP, the numbers indicate the amino acid residues included in the fusion; C has 300 residues] showed that at least the N-terminal 88 codons of the C gene were required for the enhancement of genomic RNA production to be exhibited (Fig. 1A). Examination of intensities of the genomic and SG RNA bands across this series of constructs indicated that the amount of SG RNA produced was relatively constant and

Fig. 1. Genomic and SG RNA synthesis by rubella virus and by replicons expressing GFP or RUB SP-GFP fusions. (A) Genomic diagrams of virus (Robo) or replicon (RUBrep) infectious cDNA constructs; coding regions and noncoding regions are indicated by boxes and lines, respectively. Replicons were used that expressed GFP or the following regions of the SP-ORF fused in frame with GFP: C (C-GFP); C and the N-terminal seven residues of E2 [C-E2(1-7)-GFP]; C and E2 (C-E2-GFP); or C, E2, and the N-terminal nine residues of E1 [C-E2-E1(9)-GFP], or E2 (E2-GFP). Additionally, RUBrep constructs expressing a nested set of progressively increasing N-terminal regions of the C protein fused with GFP [C(1-N)GFP; the number in parentheses is the amino acid residues of the C protein included in the fusion protein, the complete C gene is 300 residues in length] or a nested set of progressive deletions from the N-terminus of C fused with GFP [C(N-300)GFP] were employed. (B and C) Replicon-specific genomic (G) and SG RNA's were assayed by Northern blot of transfected cells using a GFP gene probe. The SG/G ratio of band intensities is given below each lane; band intensities were determined by densitometry and each ratio is the average of two independent experiments. Virus-specific RNAs in cells infected with Robo502 or F-Therien RUB were also probed using a cDNA to the SG-RNA.

that the decrease in SG/genomic RNA ratio was due to an increase in the amount of genomic RNA produced by constructs encoding a sufficient length of the C gene. As shown in Fig. 1B, analysis of replicon-specific RNAs in cells transfected with a series of eight RUBrep constructs that expressed a nested set of progressive deletions of the C



gene from the N-terminus fused with GFP revealed that the 5′ end of the C gene was required for enhancement of genomic RNA production.

We also made a series of constructs expressing fusion proteins between the C gene of the Sindbis virus (SIN), a member of the Alphavirus genus of the Togaviridae family, or 3′-truncations of the SIN C gene and GFP in both SINrep and RUBrep vectors. Enhancement of genomic RNA production in replicons of SIN by the SIN C gene was not observed (Fig. 2) and expression of the SIN C gene by RUB replicons did not lead to enhancement of genomic RNA production (Fig. 2). Thus, the phenomenon was specific to RUB and the RUB C protein.

The C gene is not able to modulate the viral RNA synthesis in trans

In the RUBrep constructs thus far analyzed, C was provided in *cis* from the replicon genome. We next conducted experiments to determine if the C gene expressed in *trans* could modulate RUBrep/GFP RNA synthesis. These experiments were performed by co-transfecting cells with (1) RUBrep/GFP transcripts and (2A) transcripts from RUBrep constructs expressing a series of C-CAT fusions containing progressively longer N-terminal regions of the C gene or (B) plasmid vectors expressing cassettes containing increasing N-terminal regions of the C gene or the complete SP-ORF under control of CMV immediate-early promoter. Modulation was detected by probing Northern blots with a GFP gene probe that will only detect RUBrep/GFP RNA species. Interestingly, when the C gene was provided in *trans*, either from another replicon (Fig. 3A) or a plasmid expression vector (data not shown), the amount of RUBrep/GFP genomic RNA was not enhanced. Expression of the C-CAT fusions from the RUBrep vector is shown in Fig. 3B.

Determination of whether the C protein or the C gene RNA is the modulating factor

Since the C gene modulates the levels of genomic and subgenomic RNAs only in *cis*, we next investigated whether the C gene RNA or protein was the moiety responsible for modulation. To this end, a series of mutations in the N-terminal eight codons of the C gene, a region necessary for modulation, were employed that either changed the RNA sequence without changing the amino acid sequence or prevented translation of the C protein. As shown in Fig. 4A, in cells transfected with RUBrep/C(2nd–8th)-GFP, in which codons 2 through 8 of the C gene were replaced with synonymous codons (Tzeng and Frey, 2003), the genomic RNA band was as intense as that synthesized by RUBrep/C-GFP. In contrast, RUBrep/C(AUA,GTC)-GFP, in which the two in-frame AUGs at codons 1 and 9 of the C gene (termed AUG1 and AUG2) were mutated to AUA and GTC, respectively, eliminating translation of the C protein, the amount of genome RNA synthesized was similar to RUBrep/GFP without enhancement. Similarly, two mutations which deleted one or two nts of codon 6 of the C gene, RUBrep/C(Δ1-nt)-GFP or RUBrep/C(Δ2-nt)-GFP, maintaining initiation at the first AUG of the C gene but preventing translation of the C-GFP fusion protein due to frame shifts (Tzeng and Frey, 2003), also lacked modulating ability and produced an amount of genome RNA similar to RUBrep/GFP. Finally, RUBrep/C(ZZΔAUG1)-GFP, in

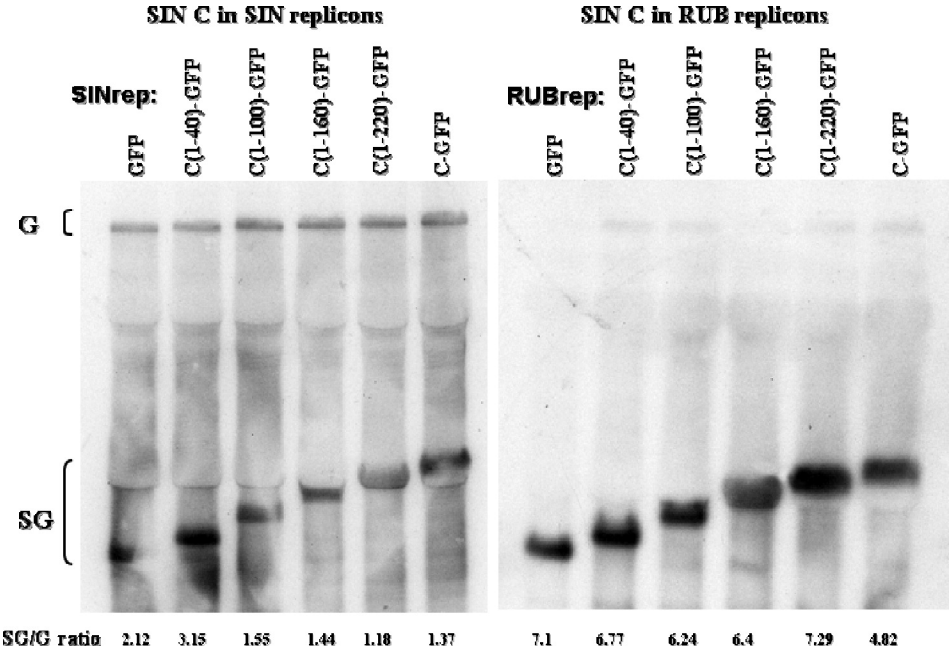


Fig. 2. Genomic and SG RNA synthesis by SIN and RUB replicons expressing the SIN C. Cells were transfected with transcripts of SIN or RUB replicons expressing GFP or a nested set of progressively increasing N-terminal regions of the SIN C protein fused with GFP and replicon-specific RNA's were detected by Northern blot.

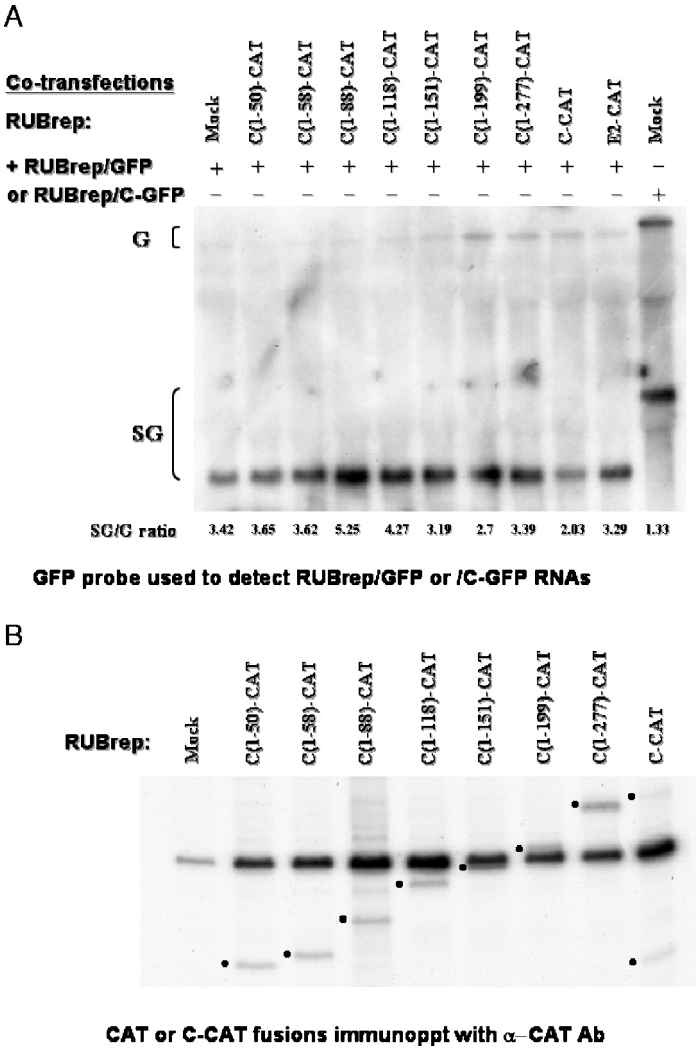


Fig. 3. RUBrep/GFP genomic and SG RNA synthesis in the presence of C provided in *trans*. (A) Northern blots were used to probe replicon-specific RNA's in extracts of cells transfected with RUBrep/GFP transcripts or co-transfected with RUBrep/GFP transcripts and transcripts from RUBrep replicons expressing a nested set of progressively increasing N-terminal regions of the C protein [C(1-8) through the complete C protein] fused in frame with the CAT gene. Extracts from RUBrep/C-GFP-transfected cells were included as a control. Since a GFP gene probe was employed, only RNAs synthesized by RUBrep/GFP or RUBrep/C-GFP were detected. (B) Companion cultures transfected with the RUBrep/C-CAT fusion series were assayed by immunoprecipitation with CAT-specific antibodies to ascertain that the C-CAT fusion proteins were produced. The expressed produced is denoted with a dot; the C-CAT fusion protein, which contains the E2 signal sequence, is partially processed into its C and CAT components, explaining the presence of two bands.

which AUG1 was replaced with UAAUAA, abrogating initiation of translation from the first AUG of the C gene, had no effect on the SG/genome RNA ratio in the transfected cells, while RUBrep/C(ZZ)-GFP, in which UAAUAA was placed immediately upstream of AUG1 (but AUG1 was maintained), the ratio of SG to genome RNA synthesized was enhanced, similar to RUBrep/C-GFP (data not shown). These results show that production of the C protein is necessary for modulation of the SG/genome RNA ratio and that the RNA sequence encoding the C gene is not a *cis*-acting element.

To test whether phosphorylation of the serine 46 of the C protein, which has been shown to be important for virus replication (Law et al., 2003), is necessary for SG/genome RNA modulation, a RUBrep/C-GFP construct in which the C phosphorylation site, S46, was mutated to A in RUBrep/C-GFP to prevent phosphorylation was employed. However, as shown in Fig. 4B, this mutation had no effect on the SG/genome RNA ratio, although the intensities of both the genomic and SG RNAs were slightly lower relative to those in RUBrep/C-GFP-transfected cells.

Discussion

The results of this study show that the RUB capsid protein can modulate the ratio of viral genome and SG RNA synthesis. The first indication of this phenomenon was comparative results from previous studies (Hemphill et al., 1988; Tzeng and Frey, 2002) leading to the observation that relatively little genome RNA relative to SG RNA was synthesized in replicon-transfected cells in comparison to

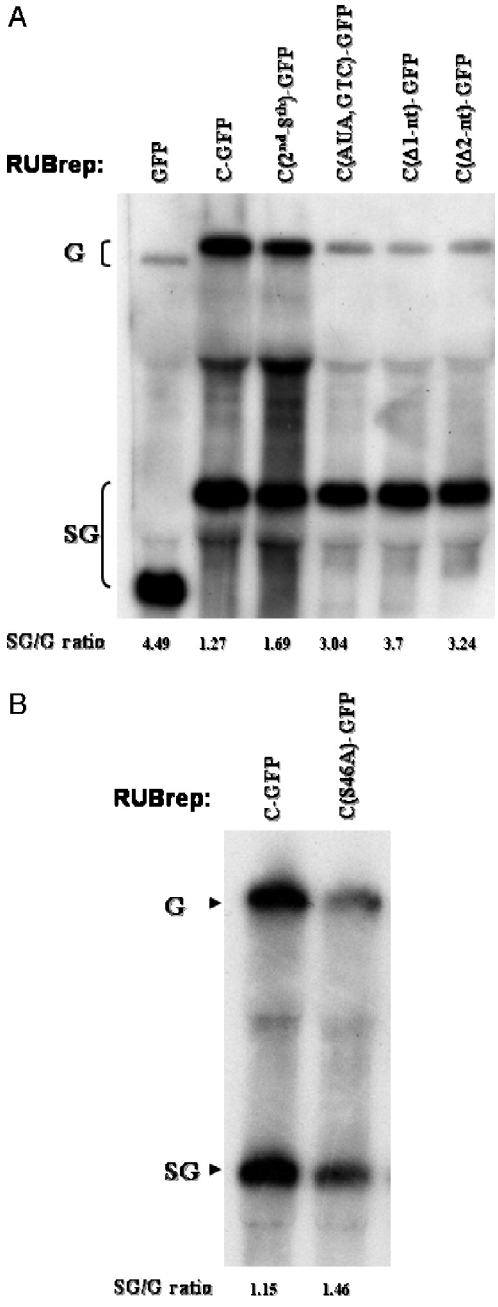


Fig. 4. Genomic and SG RNA synthesis by RUBrep/C-GFP with mutations affecting C gene sequence, C protein translation, and C protein phosphorylation. Replicon-specific G and SG RNA's were assayed by Northern blot in extracts of cells transfected with transcripts from RUBrep/GFP, RUBrep/C-GFP, or RUBrep/C-GFP constructs with mutations as indicated. (A) RNA's produced by RUBrep/GFP, RUBrep/C-GFP, RUBrep/C(2nd–8th)-GFP (codons 2–8 of the C gene mutated to synonymous codons), RUBrep/C(AUA,GTC)-GFP (AUG1 and AUG2 of the C gene mutated to AUA and GTC, respectively), RUBrep/C(Δ1nt)-GFP, and RUBrep/C(Δ2nt)-GFP (frameshift deletions of 1 or 2 nts introduced into codon 6 of the C gene). (B) RNA's produced by RUBrep/C-GFP or RUBrep/C(S46A)-GFP (phosphorylation site, S46, mutated to panel A).

virus-infected cells in which the SG/genome RNA ratio was 1.6 determined by metabolic labeling. The phenomenon was confirmed in this study and the ratio of SG to genome RNA was found to be 1.3-1.4 and 4.3, respectively, in virus-infected and replicon-transfected cells on the basis of Northern gels. Since the only difference between the virus and the replicon was the SP-ORF, we initiated this study with the hypothesis that one of the structural genes or proteins could modulate the ratio of viral genomic and SG RNA synthesis. By constructing replicons that expressed in-frame fusions of the individual structural proteins with the reporter gene GFP via the SG RNA, we are able to determine that the moiety responsible for modulation of the viral RNA synthesis was the C gene. A series of RUBrep/C-GFP fusions that contained progressive deletions of the C gene from 5′ or 3′ end showed that roughly N-terminal one-third of the C gene, including the N-terminus itself was required for the effect. We also found that the C gene of the related Alphavirus SIN did not modulate genome/SG RNA ratios in either SIN or RUB replicons and that conversely RUB C had no effect on the SG/G ratio produced by SIN replicons (data not shown). Thus, the phenomenon is specific to RUB. In an alignment between the C genes of SIN and RUB (Tzeng and Frey, 2003), it was found that the N-terminus of the SIN C protein aligned with the second Met residue of the RUB C protein (encoded by AUG2) and there was no homolog to the N-terminal eight residues of the RUB C protein (between AUG1 and AUG2) in the SIN C protein. Deletion analysis showed that this region of the RUB C protein was necessary for the modulation effect.

The modulation of the SG/genome RNA ration was observed in cells transfected with RUBrep/C-GFP, in which the C is provided in *cis* from the replicon genome. Surprisingly, we found that the C gene could not modulate SG/genome RNA synthesis when expressed in trans either from another replicon or from a plasmid expression vector. This finding made it important to determine whether the C protein or the C gene sequences functioning as *cis*-acting elements were responsible for the modulation effect. Mutations in RUBrep/C-GFP which changed the RNA sequence, but not the coding capacity, of the N-terminal eight codons by substituting synonymous codons had no effect on SG/genome RNA ratios while three mutations which prevented translation of the C protein by minimally changing the RNA sequence ablated the modulation effect. Thus, the C protein, and not the C gene RNA, was the moiety responsible for modulation. The C protein recently has been shown to complement a deletion in the P150 replicase gene (Tzeng and Frey, 2003) and rescue mutants in the 3′CAE (Chen and Icenogle, 2004). Since the former is exhibited by C protein expressed both in *cis* and in *trans*, while the latter requires *trans* expression, different activities of the C protein may be responsible for complementation/rescue of mutants and the modulation of SG/genome RNA synthesis described in this report. Since the N-terminal third of the C protein required for the modulation effect includes the phosphorylation site, residue S46, we made an S46A mutation in RUBrep/GFP to eliminate phosphorylation, but found that it did not alter the modulation effect.

The most likely explanation for the requirement of the C protein in *cis* for modulation of the SG/genome RNA ratio to occur is that the C protein translated in proximity of the replication complex, possibly from newly synthesized SG RNA, interacts directly with the replication complex, either through association with the RNA or the replicase proteins. The C protein contains a motif between residues 28 and 56, within the N-terminal third of the protein required for the modulation effect, that binds the RUB genomic RNA (Liu et al., 1996). On the other hand, it has been reported that the C protein colocalized with P150 replicase protein on tubular structures in RUB-infected cells late in infection (Kujala et al., 1999). Interestingly, the capsid protein of alfalfa mosaic virus (AMV), a member of the Alphavirus-like Superfamily as is RUB, has been shown to bind to the three genomic RNAs and that this binding is required for replication of all three of these RNAs (Bol, 1999; Jaspars, 1999). One study showed that replication of these RNAs required capsid protein provided in *cis* by RNA3, the genome RNA containing the capsid gene, and that this *cis* requirement was likely involved in plus strand RNA synthesis (Neeleman and Bol, 1999). This finding provides precedence for capsid protein functioning in plus-strand RNA synthesis as is the case with the RUB C protein modulating the ratio of the two plus-strand viral RNA species shown in this study. Since the levels of SG RNA produced by RUBrep constructs lacking or containing the C gene were relatively equivalent as detected by Northern gels, the modulation of the SG/genome RNA ratio appears to be due to an increase in the synthesis of the genome RNA or alternatively an increase in the stability of newly synthesized genome RNA. Since some translation of C gene would be necessary to trigger this upregulation in accumulation of genomic RNA, the effect could reflect a regulatory mechanism on the part of the virus to allow initial accumulation of SG RNA and translation of the virion proteins before genome RNA synthesized was maximized.

Materials and methods

Plasmids and site-directed mutagenesis

Recombinant DNA manipulations were performed essentially as described by Sambrook et al. (1989) with minor modifications. *Escherichia coli* strains JM109 and DH5α were used as bacterial hosts. Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Beverly, MA) or Roche Molecular Biochemicals (Indianapolis, IN) and used as recommended by the manufacturers. The following constructs were described previously: the RUB infectious genomic cDNA clones Robo502 and NRobo502 (Tzeng and Frey, 2002); the replicon RUBrep/GFP and RUB replicon constructs expressing a series of C-GFP fusions that contained progressive deletions of the C gene from either the 5′ or 3′ end (Tzeng and Frey, 2003); five RUBrep/C-GFP replicon constructs containing the mutations at the 5′ end of the C gene [RUBrep/C(2nd–8th)-GFP, RUBrep/C(Δ1nt)-GFP, RUBrep/C(Δ2nt)-GFP, RUBrep/C(ZZΔAUG1)-GFP, and RUBrep/C(ZZ)-GFP] (Tzeng and Frey, 2003); SIN replicon constructs expressing a fusion protein of the SIN C gene or progressive 3′ deletions of the SIN C gene and GFP (Tzeng and Frey, 2003); and plasmids containing the RUB C gene or progressive 3′ deletions of the RUB C gene under control of the human cytomegalovirus (CMV) immediate–early promoter (Tzeng and Frey, 2003).

To create RUBrep/C(S46A)-GFP in which the phosphorylated serine at residue 46 of the C protein is replaced with alanine, mutagenic upstream oligo 5′-GCC*GCGGCCG*-*C*CGCGACAGCGCGACTCC*GCA*ACCTCCGGAGATG-AC-3′ [*Not*I site (underlined) followed by nts 6619 to 6664 of the genome with GCA (italics, encoding alanine) replacing AGC (encoding serine)] and downstream oligo 5′-GTAC*TCTAGA*GCGGATGCGCCAAGGATGG-3′ [*Xba*I site (underlined) followed by a sequence complementary to nts 7324 to 7342 of the genome, a region downstream from the unique *Asc*I site at nt 7318 of the genome] were used in a PCR reaction with *Pst*I-linearized Robo502 template. The PCR reaction contained 400 ng of each oligonucleotide primer, 20 ng of linearized plasmid template, 200 μM each deoxynucleotide triphosphate, and 5 U of *Extag*DNA polymerase (PanVera/TaKaRa; Madsion, WI) in 1× buffer provided by the manufacturer in a total volume of 50 μl. The cycling protocol was 20 s at 98 °C, 20 s at 55 °C, and 1–3 min at 70 °C for 35 cycles followed by one cycle of 10 min at 72 °C. The amplified product was restricted with *Not*I and *Asc*I and ligated in a three way ligation with the *Bgl*II–*Not*I fragment from RUBrep/C-GFP (a fragment from nts 5355 to 6622 of the genome) and the *Bgl*II–*Asc*I fragment of RUBrep/C-GFP (containing the plasmid backbone plus nt 1-5355 and 7318 through the 3′ end of the replicon sequences).

To create RUBrep/C(AUA,GTC)-GFP in which AUG1 and AUG2 of the C gene were replaced with AUA and GTC, a three-round asymmetric PCR strategy was employed (Tzeng and Frey, 2002). In the first round, the mutagenic oligo 5′-CTACTACCCCCATCACC*GTC*GAG-GACCTCCAGAAGGCCC-3′ (nts 6519 to 6557 of the genome containing GTC (italicized) in place of AUG) was used to prime asymmetric amplification on a *Eco*RI-linearized RUBrep/C(AUA,AUA)-GFP template (Tzeng and Frey, 2003). In the second round, asymmetric amplification on the first round PCR product as template was primed with oligo 5′-CGCGGATCCTACTACCAGTC-CCTGCGCTGGCC-3′ (a sequence complementary to the nts 6716 to 6734 of the genome, a region downstream from the *Not*I site at nt 6622 of the genome). In the third round, the second round PCR product and oligo 5′-ACTA*ATGCA-T*CGCCCCTGTACGTGGG-3′ [*Nsi*I site (underlined) followed by nt 6387–6407 of the genome] was used to prime

PCR amplification on *Eco*RI-linearized RUBrep/C(AUA,AUA)-GFP template. The *Nsi*I–*Not*I-digested PCR amplification product was included in a three fragment ligation with the *Not*I–*Eco*RI fragment from RUBrep/C-GFP (nt 6622 through the 3′ end of the replicon sequences) and *Nsi*I–*Eco*RI fragment of NRobo502 (containing the plasmid backbone and the 5′ end of NRobo502 through the *Nsi*I site at nt 6391, the 3′ end of the NS-ORF).

To create a series of RUB replicon constructs expressing a fusion protein between the SIN C gene or progressive 3′ deletions of the SIN C gene and GFP, a standard PCR amplification was performed with *Xho*I-linearized pTE5′2J template (Hahn et al., 1992), upstream oligo 5′-CGC*GAATTCACTAGT*ATGAATAGAGGATTCTTTAAC-3′ [*Eco*RI and *Spe*I sites (underlined) followed by nts 7647 to 7667 of the SIN genome], and mutagenic downstream oligos containing an *Xba*I site followed 15∼17 nts complementary to the sequence upstream from the desired deletion site. The amplified product was restricted with *Spe*I and *Xba*I and ligated with *Xba*I linearized RUBrep/GFP.

In vitro transcription, transfection, and detection of viral RNA species and expressed fusion proteins

All plasmids were purified on CsCl isopycnic density gradients prior to use. Robo502, RUBrep, and their derivatives were linearized with *Eco*RI, while SINrep and its derivatives were linearized with *Xho*I prior to in vitro transcription, which was carried out as previously described (Tzeng and Frey, 2002). The in vitro transcription reaction mixtures were used directly for transfection without DNase treatment or phenol–chloroform extraction. Vero cells were transfected with Lipofectamine 2000 (Invitrogen; Carlsbad, CA) as previously described (Tzeng and Frey, 2002); transfection efficiency is 30–40% (Adams et al., 2003). Total cell RNA was extracted from replicon-transfected cells 12 h and 4 days post-transfection for SIN and RUB replicons, respectively, and replicon-specific RNA species present were detected by Northern blot using a NorthernMax-Gly Kit (Ambion; Houston, TX) and nick-translated, ^{32}P-labeled pGEM-GFP for replicons or pGEM-*Not*I for virus as a probe (Adams et al., 2003; Tzeng et al., 2001). For radioimmunoprecipitation, 3 days post-transfection cells were radiolabeled for 1 h, lysed, and immunoprecipitation was done with anti-CAT antibodies as described previously (Pugachev et al., 2000).

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